PRODUCTION AND CHARACTERIZATION OF PROTEIN EXTRACT FROM LEMPOYANG GINGER

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ABSTRACT

Antimicrobial protein has received tremendous attention especially by the pharmaceutical industry, which sources mainly extracted from nature such as herbs and plants. These sources are known for their nontoxic property, biodegradable, as well as easily available in the market. Among the numerous natural resources, the Lempoyang ginger which scientifically known as *Zingiber zerumbet L. Smith* had long known for its application as antifungal, anti-inflammatory, anti-ulceration and antioxidant. It is also known for treating diarrhea, coughs, asthma and some other skin diseases. Lempoyang ginger is selected for this research as very few information was published on protein of this *Zingiberaceae* family of which among all, zerumbone was widely extracted as the major component. This research aimed to produce the protein extract from Lempoyang using the expanded bed adsorption chromatography (EBAC) and then characterize it using the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The parameter that was varied for this research is the height of the bed settlement at 5cm, 6cm, 9cm and 11cm, with elution made in two-steps using 45% ethanol and 90% ethanol buffer solutions. Amberlite XAD7HP is used as adsorbents to bind the large complex protein molecules from Lempoyang feedstock with constant pump speed at 13rpm for each cycle. The eluted protein fractions are then subjected to Lowry's protein concentration determination technique before characterized for molecular weight determination using the SDS-PAGE tools. The dynamic binding capacity is determined from the 50% breakthrough of the initial feedstock solution, and the recovery ratio is determined for total protein obtained. From the results, protein concentration is higher when eluted with 45% ethanol buffer solution as protein were loosely bounded to the adsorbents while the first elution using 90% ethanol buffer solution showed a lower protein concentration. The washed protein and eluted protein fractions from EBAC which were tested on SDS-PAGE showed a protein band of eluted fraction protein band with molecular weight of 21.12 kDa.

ABSTRAK

Protein antimikrob telah menjadi tumpuan utama terutama kepada industri farmseutikal dimana sumber utamanya diperoleh secara pengekstrakan daripada tumbuh-tumbuhan dan herba. Sumber alam ini terkenal dengan ciri-ciri tidak toksik, kebolehuraian secara biologi, dan ia mudah diperoleh di pasaran. Diantara sumber yang mendapat focus adalah halia Lempoyang, dikenali dengan nama saintifiknya *Zingiber zerumbet L. Smith*, yang lama telah diketahui khasiatnya sebagai antifungi, anti-radang dan antioksida. Ia juga berupaya merawat diarrhea, batuk, asma dan beberapa masalah kulit lain. Halia Lempoyang dipilih untuk kajian ini kerana sedikit maklumat mengenai kajian berkaitan kumpulan *Zingiberaceae* dihasilkan, dan 'zerumbone' merupakan komponen protein utama yang diekstrak. Kajian ini bertujuan untuk menghasil dan mengkategori protein yang diekstrak dari Lempoyang menggunakan teknik penjerapan secara mengembang menggunakan Amberlite XAD7HP sebagai agen penjerap. Protein yang diperoleh kemudian dikategori menggunakan teknik SDS-PAGE selepas kepekatan protein diuji menggunakan teknik Lowry. Kapasiti jerapan dinamik diperoleh dari 50 peratus kemasukan sampel jus Lempoyang dan jumlah protein yang diperoleh kemudian dikira. Keputusan ujikaji menunjukkan perolehan dari 45 peratus etanol mempunyai kepekatan rotein lebih tinggi dari 90 peratus etanol kerana protein kurang terjerap pada Amberlite. Pecahan campuran protein dari basuhan dan elusi yang dikategorikan menggunakan SDS-PAGE menunjukkan sampel yang dielusi mempunyai berat molekul 21.12 kDa.

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LIST OF SYMBOLS

°C	Degree Celsius (temperature)
ϴ	Surface coverage
kDa	kilo Dalton
ml	Milliliter
cm	centimeter
nm	Nanometer
rpm	Revolution per minute
mg/ml	Milligram per milliliter
u	Superficial velocity
ε	Expanded voidage

LIST OF ABBREVIATIONS

EBAC	Expanded bed adsorption chromatography
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
GC-MS	Gas chromatography
CV	Column volume
OD	Optical density
BSA	Bovine serum albumin

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

1.1.1 Protein

The word ‘Protein’ was initially introduced by Jons Jacob Berzelius in 1838 from the Greek word (prota) which means “of primary importance” for large organic compounds with almost equivalent empirical formulas. Then, the protein study was made by James B. Sumner in 1926 showing that enzymes can be isolated and crystallized. The proof that protein has its specific structure was determined by Sir Frederick Sanger in 1955 by sequencing the first protein called insulin. Later in 1958, Max Perutz and Sir John Cowdery Kendrew came up with the three-dimensional structure of hemoglobin and myoglobin by X-ray diffraction analysis.

Proteins were classified accordingly to their functions such as enzymes, hormones, transport proteins, antibodies, receptors, signaling proteins, storage proteins etc. These compounds were also classified based on their locations in the living cells as well as the posttranslational modifications such as native proteins, cleaved proteins, prions and others. Proteins structural organization can be identified as primary, secondary, ternary and quaternary based on their folding and the protein interactions.

1.1.2 Antibacterial compound in *Zingiber zerumbet L. Smith*

An antifungal protein is one type of widely known protein that function to treat or destroy the activity of fungi which causes diseases or worst, fatalities in living cells such as plant and animal, including human. In spices such as the ginger plants namely the Zingiberaceae was widely used as medicine formulations for relieving stomachache, macerated in alcohol which was regarded as tonic and depurative. Zerumbone contains proteins functioning as antifungal agent, and was isolated from Zingiber Zerumbet (L) Smith having unique structure with a cross-conjugated ketone in an 11-membered ring displaying selectivity in cytotoxic characteristics towards cancer cell lines and normal cell lines. Throughout studies and experiments made using zerumbone, the compound showed an antiproliferative activity upon HepG2 cells as well as of non-malignant Chang Liver and MDBK cell lines (SA Sharifah Sakinah, S Tri Handayani and LP Azimahtol Hawariah, 2007). Zerumbone which also expressed as ZER was also found to suppress tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation is some potent manner. The compound was also indicated to be having distinct potentials for usage in anti-inflammation, chemoprevention and chemotherapy strategies (M. Akira et al., 2002).

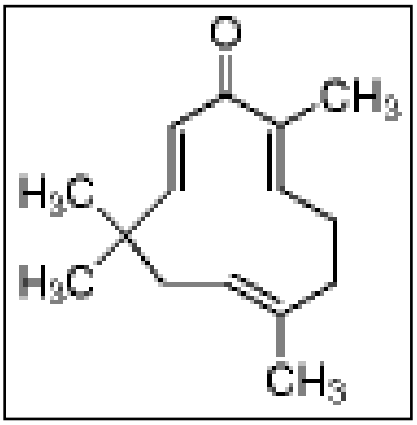

Figure 1.1: Zerumbone

1.1.3 Expanded Bed Adsorption Chromatography (EBAC)

Various methods were proposed and commercially used for protein production process including the liquid-liquid separation, packed bed adsorption, hydrodistillation and many others. Expanded bed adsorption (EBA) method was lately recognized as a tool used in advantage of yielding a higher concentrated product form the unclarified feedstock. It was considered an advantageous tool for this purpose as EBA process provide a less processing time and labor as it combines the clarification, initial purification and concentration step in one unit operation, hence reducing the cost of operations.

The EBA requires five (5) main steps which include the bed expansion stabilization, feedstock loading, washing of the bed column, elution with elution buffer, and finally regeneration of the bed. The bed needed to be stabilized before sample loading to determine the effective bed expansion ratio, and once sample is loaded into the column, the flowrate was to be measured in order to determine the effective loading flowrate. All these steps were carried out in an upwards direction as the adsorbents used for this research was Amberlite XAD7HP, which contain different sizes of molecules, and therefore, by applying the upwards motions, the bed expansion will be uniformly distributed accordingly to the sizes of the adsorbents. Also, this kind of load flow motion provided a better adsorption compared to packed bed adsorption.

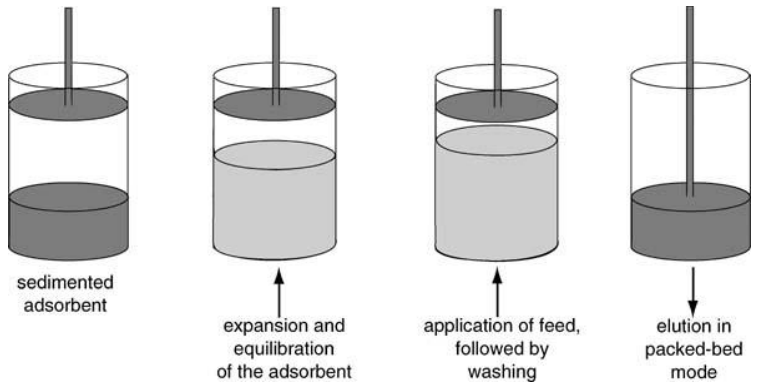

Figure 1.2: The expanded adsorption chromatography flow technique.

1.1.4 Adsorbents for Protein Adsorption: Amberlite XAD7HP

Amberlite XAD7HP was one type of adsorbent that showed capability of recovering plant extracts. Its large pores size was an advantage for adsorbing protein molecule in plant extracts or other natural sources compared to other adsorbents such as Amberlite XAD16 or Amberlite XAD1180 which might damage the peptides and protein molecules during adsorption. This acrylic polymer also showed ability to be performed in elution or regeneration step by using solvents, buffer or steam depending on the molecule type under consideration.

Amberlite XAD7HP was supplied in white insoluble beads, patented in macroreticular structure with aliphatic nature which able to adsorb non polar compounds from aqueous systems and also adsorbing the polar compounds from non-polar solvents. During shipment, this polymeric adsorbent was inhibited with NaCl and Na2CO3 salts to retard the bacterial growth. These salts were to be washed when the adsorbent was prior to use and the washing was suggested at a linear flowrate of 5-10m/h up to the required level.

1.2 PROBLEM STATEMENTS

The pharmaceutical industry has been developing methods to increase the production of antimicrobial protein from nature as it is seen as an advantage of using the biodegradable compounds which is non-toxic to human body and at the same times effective for treating diseases. Among all nature's products, the Lempoyang ginger has received tremendous attention in extracting and purifying the antimicrobial protein namely zerumbone mostly from the plant's rhizomes. However, due to very limited research being made on this plant, and very few information regarding this antimicrobial protein being published in journals, researchers has come out with various method for extracting this protein such as using the extraction and evaporation technique (2011), hydrodistillation (2009) as well as using the ion-exchange chromatography (2005).

1.3 OBJECTIVES

This research is purposely carried out in order to produce the protein extract from Lempoyang ginger, scientifically named *Zingiber zerumbet L. Smith*, by applying the expanded bed adsorption chromatography (EBAC) technique with Amberlite XAD7HP was selected as the adsorbents for this research. This research is also to characterize the protein extracted from EBAC according to their respective molecular weight by using the sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique, in order to determine the presence of antimicrobial protein namely zerumbone in the sample feedstock. At the end of the research, the main aim will focus on the parameters that can give a higher yield of protein concentration by varying the height of bed settlement and buffer concentrations.

1.4 SCOPES OF STUDY

The research is focused on the scopes as listed below in order to achieve the objectives outlined:

i) To study the extraction of protein from Lempoyang ginger using the expanded bed adsorption chromatography technique of Fastline 10 Column and Amberlite XAD7HP as the adsorbents.

ii) To determine the optimal settled bed height that will give a higher yield of protein from adsorption process of expanded bed adsorption chromatography technique (EBAC).

iii) To determine the presence of antimicrobial protein of zerumbone by characterization of eluted sample from expanded bed adsorption chromatography (EBAC) using the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique.

1.5 SIGNIFICANCE OF STUDY

This research applied the expanded bed adsorption chromatography (EBAC) technique for the purpose of protein extraction from unclarified feedstock. The uniqueness of this technique is that the feedstock loaded into the column is not necessarily be clarified as this EBA combines all three steps of feedstock clarification, concentration and initial purification within one unit operation. This combination is a major advantage among other techniques as it capable of reducing the cost of operation and production, reducing the time used as well as easy to be applied in industry.

Generally, protein concentration is determined using Lowry's method. However, the concentration value read is of total protein present in the sample. Hence, sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to characterize the respective protein accordingly to its individual molecular weight, which also seen as a more accurate method for determining the presence of desired protein in the sample.

CHAPTER 2

LITERATURE REVIEWS

2.1 *Zingiber zerumbet L. Smith*

Zingiber zerumbet (L) Smith is commonly known as wild ginger, and in most Asian countries; this ginger is called Lempoyang Ginger or "Shampoo Ginger". It is part of the Zingiberaceae family and originated from Indonesia which later distributed in Malaysia, Bangladesh, India, Nepal, Sri Lanka and other parts of the world including Hawaii. *Z. zerumbet* has its unique properties which act as an anti-inflammatory, anti-ulceration, anti-oxidant and anti-microbial agent, and it has been long used for medical treatment easing diarrhea, ear inflammation, swelling, sores, relieving rheumatic pain, etc. It usually can be found grown in the secondary forests or at the village edges and some also planted this ginger in gardens throughout the tropics. Zerumbone and α–caryophyllene are the major chemical compounds found in essential oil from the extraction of leaves and rhizomes of *Z. zerumbet*. The zerumbone oil after purified and processed was used as anticancer bioactive compound for treating cancers such as breast cancer and cervix cancer.

Figure 2.1: *Zingiber zerumbet (L.) Smith* plant.

2.2 ISOLATION AND PROTEIN PRODUCTION FROM *Z. zerumbet*

In expanded bed adsorption process, the adsorbents used need to be of size in range of 50 to 400 microns. The larger adsorbents were sedimented to the bottom of the column while the smaller particles at the top of the sediment. In extraction and purification of antifungal protein in Lempoyang Ginger, variant types of techniques were used such as Amberlite as adsorbent in Expanded Bed Adsorption (EBA) technique, Tris-HCl buffer which was applied to the DEAE-cellulose and affinity chromatography process, as well as using the hydrodistillation technique and modified Clevenger-type glass apparatus for extraction of essential oil with analysis of antifungal compound using GC-MS. However, only adsorption process showed a simpler purification technique compared to the others, hence, this technique was proposed to be applied in the protein purification industry especially the pharmaceutical industries which minimize the production cost due to single purification step of adsorption process.

2.3 ADSORPTION ISOTHERMS

2.3.1 Langmuir Adsorption Isotherm

The Adsorption of Adsorbate on Adsorbent surface isotherm was made by Irving Langmuir in 1916, and was awarded Nobel Prize in 1932 for the investigation of surface chemistry. The isotherm required three assumptions describing the adsorption of adsorbate to the surface of the adsorbents. First was that the adsorbent surface having contact to the solution containing adsorbate which was strongly attached to the adsorbent surface, secondly was the surface of the adsorbent having specific number of sites allowing adsorption of the solute molecules to the surface, and third was that the adsorption occurred in monolayer, which means the molecules attachments only occurred in one layer of adsorption (Duff, David G., Ross Sheina M. C. and Vaughan, D. Huw, 1988). The following chemical reactions represent the monolayer adsorption process which later, explain the isotherm.

$$\mathbf{A + S \leftrightarrow AS}$$

where AS represent the solute molecules bound to the surface site of S. Given the equilibrium constant consisting of the concentration of A and S expressed in units such as mol/cm2:

$$\mathbf{K} = \frac{[\mathbf{AS}]}{[\mathbf{A}][\mathbf{S}]} \quad (1)$$

The complete isotherm considered in terms of the surface coverage, Θ, was defined by the fraction of adsorption sites to which a solute molecule has attached to the surface. The unattached sites were expressed as (1- Θ) which related to the concentration by:

$$\frac{[\mathbf{AS}]}{[\mathbf{A}][\mathbf{S}]} = \frac{\boldsymbol{\theta}}{\mathbf{1}-\boldsymbol{\theta}} \quad (2)$$

The isotherm was expressed by replacing [A] as C, and the complete isotherm expression was achieved:

$$\mathbf{K_C} = \frac{\boldsymbol{\theta}}{\mathbf{C}(\mathbf{1}-\boldsymbol{\theta})} \quad \text{where} \quad \Theta = \frac{\mathbf{K}}{\mathbf{1}-\mathbf{K}} \quad (3)$$

2.3.2 Freundlich Isotherm

The isotherm of Freundlich was proposed due to lower concentration of substance used, also as an alternative of determining the adsorption process developed by Herbert F. Freundlich. The Freundlich Isotherm was expressed as below, with k and n as parameters being empirically determined by plotting log Y vs. log C, using: Y = kC 1/n.

(Duff, David G., Ross Sheina M. C. and Vaughan, D. Huw, 1988).

2.4 EXPANDED BED ADSORPTION CHROMATOGRAPHY (EBAC) PROCESS

Expanded Bed Adsorption (EBA) represents the most exciting development in the field of bimolecular separations since the introduction of packed bed chromatography in the 1950s and it was applied for adsorption of streptomycin in 1950s and novobiocin in the 1970s (Richard J. P. Cannell). However, a stabilized expanded bed adsorption was developed in early 1990s with approach being used in processing of whole cell mammalian cell culture broth as well as successful in affinity chromatography application (John M. Walker & Ralph Rapley). Expanded bed adsorption was a one unit operation process accomplishing the removal of whole cell and cell debris (clarification step), concentration and initial purification of target protein from the crude extract loaded into the bed column (Michael John Lewis).

There are five main steps in adsorption process using the expanded bed technique. First was the expansion of packed bed into expanded bed using a particulate-free liquid. Secondly was the feeding of the sample solution containing particulate matter into the column until the adsorbent get saturated with the product. Then in third step, the column was upwashed as to remove the remaining particulate in the void of the bed. Fourth step was converting the expanded bed to packed bed state by gravity settling or reversed flow which product eluted from the adsorbents. Finally at fifth step of adsorption, the adsorbents were cleaned to remove the strongly bound impurities and re-equilibration procedure was made for next batch. Prior to 1980‟s, the expanded bed technique faced obstacles in determining the specific adsorbents being designed for the expanded bed procedures, which also the major obstacle for the technology to be implemented in both laboratory and industrial scale (Richard J. P. Cannell).

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The design methods for producing and characterizing the protein extract was initially made prior for carrying out the process. In the methods designed, all material, chemicals and equipments will be listed accordingly to the step of producing the protein extract using the expanded bed adsorption chromatography (EBAC) technique, and characterization of the protein using the sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique. The experiment was done in the bioprocess laboratory of Chemical Engineering in University Malaysia Pahang (UMP).

3.2 MATERIAL AND EQUIPMENTS

The main raw material used for this research project is the Lempoyang ginger rhizomes which were purchased from a market in Johor. The protein concentration determination will be using chemicals of Lowry's reagent, Folin-Ciocalteu reagent and the SDS-PAGE solutions.

3.2.1 Warring/Juice blender

The blender is used to separate the crude ginger juice from ginger biomass such as the ginger root fibers. The blender is of automatic setting, therefore the speed is kept constant for each juice extraction of ginger blending.

3.2.2 Vacuum filter

The vacuum filter was used to remove the remaining large solid particles in crude juice. A suspended-solid (SS) filter paper was used for the filtration as normal laboratory filter paper will take a longer time to produce an initial clarification of the crude juice.

Figure 3.1: Vacuum pump.

3.2.3 Expanded Bed Fastline 10 Column

The adsorption of protein compounds was carried out using the expanded bed of Fastline 10 Column at range of 5 to 12 cm of settled bed heights. Amberlite XAD7HP was used as adsorbents and the settled bed heights will be according to the settlement of this adsorbent in column.

3.2.4 UV-Vis Spectrophotometer

The UV-Vis spectrophotometer is used to determine the protein concentration of eluted fractions from EBAC. The ultraviolet wavelength determines the absorbance of protein present.

Figure 3.2: The UV-Vis spectrophotometer.

3.2.5 The SDS-PAGE Equipment

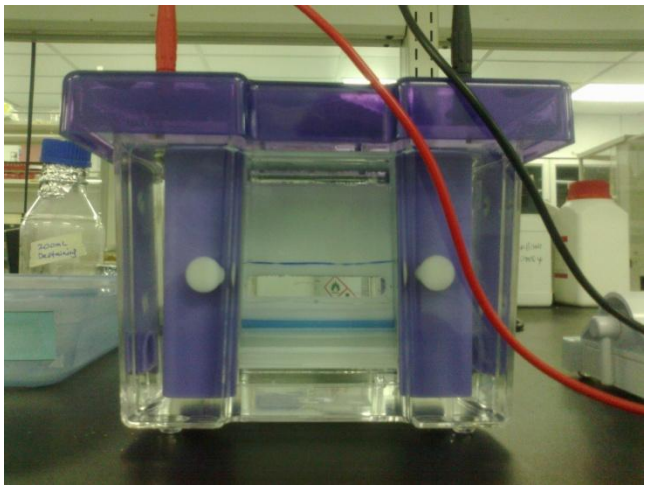

Figure 3.3: The SDS-PAGE tool.